Biochem. J. (1977) **163**, 303–307
Printed in Great Britain

Evidence that Latent Collagenases are Enzyme–Inhibitor Complexes

By ANTHONY SELLERS, ELIZABETH CARTWRIGHT, GILLIAN MURPHY and JOHN J. REYNOLDS
Cell Physiology Department, Strangeways Research Laboratory, Cambridge CB1 4RN, U.K.

(*Received* 29 *November* 1976)

Specific collagenase from the culture media of various rabbit tissues and cells exists in active and latent forms. Latent collagenase is most effectively activated with 4-aminophenylmercuric acetate, a thiol-blocking reagent, strongly suggesting that latent forms are enzyme–inhibitor complexes. A collagenase inhibitor from bone cultures, which may be closely related to the inhibitor of such latent enzyme complexes, was partially characterized.

Collagen is the major structural protein of connective tissues, and to understand its degradation in physiological and pathological conditions much research has been directed towards characterizing collagenases which act on the triple helix of collagen (Harris & Krane, 1974*a*,*b*,*c*; Harris & Cartwright, 1977). Many investigators, including ourselves, have attempted to define the stimuli controlling the synthesis and secretion of collagenase by connective-tissue cells, but it is clear that the extracellular control of the activity of collagenase is also of great importance. Two mechanisms have been proposed for this control: (*a*) that there is an extracellular conversion step from latent proenzyme (procollagenase) into active enzyme (Vaes, 1972; Harper & Gross, 1972; Kruze & Wojtecka, 1972; Hook *et al.*, 1973), and (*b*) that there is secretion by cells of active collagenase, but the activity is modulated by inhibitors (Bauer *et al.*, 1972, 1975; Nagai, 1973; McCroskery *et al.*, 1975; Woolley *et al.*, 1976). The experimental basis for both of these hypothetical mechanisms is the presence of latent forms of collagenase in the culture fluid of cells and tissues. Reports have described the activation of latent collagenases by proteinases, especially trypsin (Vaes, 1972; Hook *et al.*, 1973; Bauer *et al.*, 1975; Birkedal-Hansen *et al.*, 1976; Dayer *et al.*, 1976; Horwitz & Crystal, 1976), but sodium thiocyanate (Nagai, 1973; Birkedal-Hansen *et al.*, 1976) has also been used.

Previous work (Werb & Burleigh, 1974; Werb & Reynolds, 1974) indicated that collagenase activity in the culture medium of rabbit synovial fibroblasts could be enhanced by 4-chloromercuribenzoate. This encouraged us to investigate the ability of thiol-blocking reagents to activate latent collagenases and provide evidence that latency is the result of these enzymes existing as enzyme–inhibitor complexes.

Materials and Methods

Materials

4-Aminophenylmercuric acetate was purchased from Aldrich Chemical Co., Wembley, Middx. HA0 1PY, U.K. Bovine pancreatic trypsin was purchased from Miles–Seravac (Pty.) Ltd., Stoke Poges, Slough SL2 4LY, Berks., U.K. Ultrogels AcA 44 and AcA 54 were purchased from LKB, South Croydon, Surrey CR2 8YD, U.K. All other materials have been described elsewhere (Werb & Burleigh, 1974).

Methods

Culture techniques. Methods for the growth of tissues and cells *in vitro* for the production of collagenase were described previously (Werb & Burleigh, 1974; Werb & Reynolds, 1974). Bone explants (parietal bones from rabbit foetus at 22–29 days of gestation; one bone per dish containing 1.5ml of medium) were cultured in a modified form of BGJ medium (Reynolds, 1976) supplemented with 5% (v/v) heat-treated rabbit serum. This medium contained no active α_2-macroglobulin or other collagenase inhibitors.

Collagenase assay. This was by measurement of the release of ^{14}C-labelled peptides from reconstituted fibrils of radioactive rat skin collagen, and the results are expressed in terms of percentage gel lysis (Werb & Burleigh, 1974).

Activation of latent enzymes. Preliminary experiments were undertaken to determine the concentrations of trypsin and 4-aminophenylmercuric acetate required for maximal activation of each batch of medium. The procedures for activation were as follows:

(*a*) With trypsin. Culture fluid was incubated with

bovine pancreatic trypsin (0.2–0.5mg/ml of activation mixture) either for 30min at 4°C (rabbit bone, mouse bone and human synovium) or for 1min at 25°C (rabbit uterus, skin and synovial cells). After incubation soya-bean trypsin inhibitor (fivefold excess w/w) was added before the collagenase assay.

(*b*) With 4-aminophenylmercuric acetate. Stock solutions (10mM) were prepared daily and added directly to the fibril assay to give a final concentration of 0.5–1.0mM (the experiments in Table 1 below). Alternatively, 4-aminophenylmercuric acetate was preincubated with enzyme for 2–4h at 35°C, cooled to 4°C and chromatographed on Ultrogel AcA 44 to remove the excess of 4-aminophenylmercuric acetate (the experiments in Tables 2 and 3 below).

Inhibition of collagenase by the bone collagenase inhibitor. Media from the early days of foetal rabbit bone culture, which contained negligible latent or active collagenase, were used as the source of collagenase inhibitor (see the Results section). The quantity of inhibitory medium added to all the incubations in Table 2 was initially established by measurement of the volume required to give approx. 75% inhibition of 0.05 unit of active rabbit bone collagenase (1 unit of collagenase hydrolyses 1 μg of rat skin collagen/min at 35°C) and represents 50% gel lysis in a 20h incubation.

Chromatography for molecular-weight determinations. Conditions were similar to those described (Werb & Reynolds, 1975), except that Ultrogels AcA 44 and AcA54 were used in place of Sephadex. Calibrated columns were run in 0.2M-NaCl, or in 1M-NaCl to decrease anomalous aggregation and adsorption on to the gel matrix. Molecular weights were calculated by using the method and standards (bovine serum albumin, ovalbumin, soya-bean trypsin inhibitor and cytochrome *c*) of Andrews (1964).

Results

Activation of latent collagenase

We examined the culture media from a variety of rabbit tissues and cells. The culture media from nongravid uterus had no active collagenase during the first 10 days (changes of medium daily), but large amounts of latent enzyme were present after a lag of 4–5 days. The culture media from rabbit bone and skin contained both latent and active enzyme. The proportion of latent to active collagenase produced by synovial fibroblasts is variable: in the present experiments, little or no active collagenase was produced by the cells, but previously we found that rabbit synovial-cell lines could produce large amounts of active enzyme (Werb & Burleigh, 1974; Harris *et al.*, 1975) with little latent enzyme.

The latent collagenase of all the media described above could be activated by treatment with trypsin. Alternatively, the inclusion of 4-chloromercuribenzoate or other thiol-blocking reagents in the collagenase assay activated latent forms, but to varying extents. In a comparative experiment using medium from rabbit skin cultures, 4-aminophenylmercuric acetate (0.7mM) gave 100% activation of latent enzyme as compared with trypsin, whereas 4-chloromercuribenzoate (0.7mM) gave 13%, 4-hydroxymercuribenzenesulphonate (1.0mM) gave 38%, sodium mersalylate (0.7mM) gave 16%, and sodium tetrathionate (0.7mM) gave 11% activation respectively. The most effective reagent was always 4-aminophenylmercuric acetate. 4-Aminophenylmercuric acetate had no effect on collagen; no difference was observed either if culture fluids were preincubated with 4-aminophenylmercuric acetate and the excess was removed by dialysis, or if the reagent was included in the assay.

We compared the activation of latent collagenase from rabbit tissues and cells by either 4-aminophenylmercuric acetate or trypsin on the same batches of medium (Table 1). 4-Aminophenylmercuric acetate was always at least as effective as trypsin in activating latent collagenase, but the effects of the two treatments were never additive. We obtained similar results with latent collagenases in culture fluids of human synovium and mouse bone; T. Cawston & J. Tyler (personal communication) have also obtained greatest activation of latent pig collagenase with 4-aminophenylmercuric acetate. Activation of latent collagenase from the above sources by either trypsin or 4-aminophenylmercuric acetate gave enzymes capable of cleaving collagen in solution at 25°C into the characteristic three-quarters and one-quarter-length fragments (Werb & Reynolds, 1975).

Collagenase inhibitor from rabbit bone

Foetal rabbit bone explants were maintained in culture for several weeks with changes of medium every 2 days. After an initial lag varying from 1 to 8 days collagenase was released into the medium. The enzyme was first present in a latent form; active collagenase appeared in the medium in the latter part of the culture period, and the amount of it increased as the culture progressed (up to 80% of the total). During the lag period, before the production of latent collagenase, we detected an inhibitor of collagenase in the medium. As the amount of latent collagenase released into the culture medium increased, the inhibitory activity fell to undetectable values. The inhibitor blocked the action of collagenase either found in the active form or that from latent enzyme activated by 4-aminophenylmercuric acetate (Table 2). The inhibitor was usually not effective on collagenases produced by activation with trypsin. Estimates of the molecular weight of the inhibitor on Ultrogel AcA 44 and AcA 54 gave a value of 30000.

Table 1. *Comparison of the activation of latent collagenases by 4-aminophenylmercuric acetate and trypsin*

Details are given under 'Methods' and in the Results section. The results are expressed as percentage gel lysis for each comparative set of experiments on different batches of media. Incubations were carried out for 20h at 35°C in a final volume of 350μl, and sufficient medium containing enzyme was used such that activated enzyme (maximal conditions) would give gel lysis within the linear part of the assay.

Source of collagenase	Expt. no.	Amount of medium (μl/tube)	Gel lysis (%) No activation	Activated by 4-aminophenylmercuric acetate	Activated by trypsin	Activated by trypsin and 4-aminophenylmercuric acetate
Rabbit bone	1	60	0	70	69	67
	2	80	0	45	40	42
	3	60	0	52	48	53
Rabbit skin	1	50	8	52	45	46
	2	20	10	65	60	60
	3	50	5	35	35	37
Rabbit uterus	1	50	0	63	63	64
	2	40	0	70	67	65
	3	50	0	52	46	48
Rabbit synovial fibroblasts	1	20	0	40	39	35
	2	100	0	30	28	25
	3	80	0	31	28	28

Table 2. *Inhibition of rabbit collagenase by the rabbit bone inhibitor*

In each experiment, samples of foetal rabbit bone culture media (40–60μl) containing the same amount of inhibitory activity towards active rabbit bone enzyme (see under 'Methods') were incubated in a final volume of 350μl with either active or activated collagenases from the sources indicated below for 20h at 35°C in the standard fibril assay. Excess of 4-aminophenylmercuric acetate was removed from activated enzyme by either dialysis or gel filtration. Results are expressed as percentage gel lysis ± S.E.M. for the numbers of experiments given in parentheses.

Enzyme type and source	Amount of enzyme (μl/assay)	Gel lysis (%) Control	+Inhibitor	Inhibition (%)
Active enzyme				
Bone (8)	40–80	49 ± 10.5	13 ± 5.2	73
Skin (3)	60–80	45 ± 5.0	30 ± 7.5	33
Trypsin-activated enzyme				
Bone (3)	20–40	45 ± 3.5	39 ± 5.0	13
Skin (3)	15–50	60 ± 12.5	65 ± 12	0
Uterus (3)	40–100	50 ± 5.0	45 ± 6.0	10
4-Aminophenylmercuric acetate-activated enzyme				
Bone (3)	60–80	47 ± 2.5	16 ± 0.5	70
Uterus (3)	20–60	55 ± 4.0	35 ± 1.5	36

Heat treatment or incubation with either trypsin or chymotrypsin abolished the activity of the inhibitor. Bone explants either cultured in the presence of cycloheximide (0.2mM) or frozen and thawed three times did not release inhibitor into the culture fluid, indicating that the inhibitor was synthesized by the bone explants.

Apparent molecular weights of active, latent and activated rabbit collagenases

By use of calibrated Ultrogel AcA 44 columns we estimated the molecular weights of active, latent and activated collagenases from rabbit bone and uterus (Table 3). Active rabbit bone collagenase had an apparent mol.wt. of 28000, which is in close agreement with the value for collagenase from rabbit synovial fibroblasts (Werb & Reynolds, 1975). Activation of latent rabbit bone and rabbit uterine collagenase by either trypsin or 4-aminophenylmercuric acetate produced decreases in mol.wt. in the range 8000–15000.

Of particular importance was the observation that the combination of 4-aminophenylmercuric acetate-activated uterine enzyme with the bone inhibitor

Table 3. *Molecular weights of active, latent and activated forms of rabbit collagenase and of activated uterine collagenase complexed with bone inhibitor, by chromatography on Ultrogel AcA 44*

Abbreviations: HS, 1 M-NaCl in buffer; LS, 0.2 M-NaCl in buffer; n.d., not determined; —, never detected.

Collagenase		Apparent mol.wt.	
		Enzyme from rabbit uterus cultures	Enzyme from rabbit bone cultures
Active collagenase	(LS)	—	28000
Latent collagenase	(HS)	39000	44000
	(LS)	42000	37000
Collagenase activated with 4-aminophenylmercuric acetate	(HS)	26000	35000
	(LS)	n.d.	28000
Collagenase activated with trypsin	(LS)	27000	26000
Collagenase activated with 4-aminophenylmercuric acetate and complexed with bone inhibitor*	(HS)	40000	n.d.

* 4-Aminophenylmercuric acetate-activated uterine enzyme (2ml; 2.5units/ml) eluted from an AcA 44 column was incubated for 4h at 35°C with sufficient medium containing bone inhibitor (4ml) to ensure that 75% of the enzyme activity was inhibited.

produced a latent enzyme complex which, after chromatograhy on Ultrogel AcA 44, could be re-activated by either 4-aminophenylmercuric acetate or trypsin. The apparent mol.wt. of this complex was 40000, similar to that of latent uterine enzyme.

Discussion

Our data strongly suggest that latent collagenases are enzyme–inhibitor complexes because (*a*) 4-aminophenylmercuric acetate activates latent enzyme as effectively as does enzymic treatment, and (*b*) bone explants synthesize a collagenase inhibitor whose properties suggest that it is closely related to the inhibitor of such complexes.

With the rabbit bone and uterine enzymes, the conversion of latent into active collagenase results in a decrease in mol.wt. of 8000–15000, giving collagenase of molecular weight similar to that of the naturally occurring active enzyme. These data are in agreement with the decrease in mol.wt. of 10000–20000 reported for the trypsin-activated latent enzymes from other sources (Vaes, 1972; Harper & Gross, 1972; Birkedal-Hansen *et al.*, 1976). We think that 4-aminophenylmercuric acetate activates by dissociation of the complex, whereas trypsin acts by preferential degradation of the inhibitor portion of the complex. We have no evidence for the action of 4-aminophenylmercuric acetate on an intermediate activator system as described by Vaes (1972). We think it significant that the gradual disappearance of inhibitory activity from the media of bone explants is paralleled first by the appearance of latent collagenase in the media and eventually by the detection of active enzyme.

The collagenase inhibitor from rabbit bone seems to differ in both molecular weight and binding properties from the tissue inhibitors described by McCroskery *et al.* (1975) and Bauer *et al.* (1975), or the β_1 serum anti-collagenase described by Woolley *et al.* (1976). The inhibitor combines with the 4-aminophenylmercuric acetate-activated uterine enzyme to yield a latent enzyme with properties similar to those of the latent collagenase produced by the uterus in culture. The combination of inhibitor (mol.wt. 30000) with active collagenase (mol.wt. 30000) yields latent enzyme of approx. mol.wt. 40000. Whether this discrepancy can be accounted for either by considerable conformational changes on formation of the complex or by the combination of enzyme with one unit of a polymeric inhibitor remains speculative. It is difficult, however, to be certain of the precise molecular weights of collagenase measured by gel filtration (Werb & Burleigh, 1974); when much larger amounts of materials are available the molecular-weight changes must be confirmed by sodium dodecyl sulphate/polyacrylamide-gel electrophoresis.

If our proposal can be further substantiated, the existence of enzyme–inhibitor complexes would necessitate the reinterpretation of previous work attempting to correlate enzyme synthesis and secretion with collagen breakdown (Harris & Krane, 1974*a*,*b*,*c*; Harris & Cartwright, 1977). It will therefore be very important to elucidate the role of the inhibitor and to determine whether changes in its synthesis or degradation are important in the regulation of collagenase activity. Currently the favoured view is that the latent collagenases have to be activated *in vivo* to liberate active enzyme, but it is pertinent to consider the possibility that the latent enzymes instead represent collagenases that are destined for removal from the tissue, having completed their action and then complexed with inhibitor.

This work was supported by grants from the Medical Research Council and the Smith, Kline and French Foundation. E. C. is a Beit Fellow. We thank Mrs. Wendy Beard and Mr. Gary Dew for excellent technical assistance.

References

Andrews, P. (1964) *Biochem. J.* **91**, 222–233
Bauer, E. A., Eisen, A. Z. & Jeffrey, J. J. (1972) *J. Invest. Dermatol.* **59**, 50–55
Bauer, E. A., Stricklin, G. P., Jeffrey, J. J. & Eisen, A. Z. (1975) *Biochem. Biophys. Res. Commun.* **63**, 172–178
Birkedal-Hansen, H., Cobb, C. M., Taylor, R. E. & Fullmer, H. M. (1976) *Biochim. Biophys. Acta* **429**, 229–238
Dayer, J. M., Krane, S. M., Russell, R. G. G. & Robinson, D. R. (1976) *Proc. Natl. Acad. Sci. U.S.A.* **73**, 945–949
Harper, E. & Gross, J. (1972) *Biochem. Biophys. Res. Commun.* **48**, 1147–1152
Harris, E. D. & Cartwright, E. C. (1977) in *Proteinases of Mammalian Cells and Tissues* (Barrett, A. J., ed.), pp. 249–283, North-Holland, Amsterdam
Harris, E. D. & Krane, S. M. (1974*a*) *N. Engl. J. Med.* **291**, 557–563
Harris, E. D. & Krane, S. M. (1974*b*) *N. Engl. J. Med.* **291**, 605–609
Harris, E. D. & Krane, S. M. (1974*c*) *N. Engl. J. Med.* **291**, 652–661
Harris, E. D., Reynolds, J. J. & Werb, Z. (1975) *Nature (London)* **257**, 243–244
Hook, R. M., Hook, C. W. & Brown, S. I. (1973) *Invest. Ophthalmol.* **12**, 771–776
Horwitz, A. L. & Crystal, R. G. (1976) *Biochem. Biophys. Res. Commun.* **69**, 296–303
Kruze, D. & Wojtecka, E. (1972) *Biochim. Biophys. Acta* **285**, 436–446
McCroskery, P. A., Richards, J. F. & Harris, E. D. (1975) *Biochem. J.* **152**, 131–142
Nagai, Y. (1973) *Mol. Cell. Biochem.* **1**, 137–145
Reynolds, J. J. (1976) in *Organ Culture in Biomedical Research* (Balls, M. & Monnickendam, M. A., eds.), pp. 355–366, Cambridge University Press, Cambridge
Vaes, G. (1972) *Biochem. J.* **126**, 275–289
Werb, Z. & Burleigh, M. C. (1974) *Biochem. J.* **137**, 373–385
Werb, Z. & Reynolds, J. J. (1974) *J. Exp. Med.* **140**, 1482–1497
Werb, Z. & Reynolds, J. J. (1975) *Biochem. J.* **151**, 645–653
Woolley, D. E., Roberts, D. R. & Evanson, J. M. (1976) *Nature (London)* **261**, 325–327